**3,17 β -Bis-sulfamoyloxy-2-methoxyestra-1,3,5(10)-triene (STX140) and
Non-Steroidal Sulfamate Derivatives Inhibit Carbonic Anhydrase IX:
Structure-Activity Optimization for Isoform Selectivity**

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Keywords: Carbonic Anhydrase IX, Inhibitors, Cancer, Sulfamates

Abstract

STX140, a bis-sulfamate derivative of the endogenous steroid 2-methoxyestradiol, has shown promising anti-cancer potency both *in vitro* and *in vivo*, with excellent bioavailability. Its activity against taxane-resistant xenografts make it a potential drug candidate against triple negative breast cancer (TNBC). These properties are linked to the ability of STX140 to act in a multi-targeting fashion *in vivo* as a microtubule disruptor, leading to cell cycle arrest and with both pro-apoptotic and anti-angiogenic activities. Carbonic Anhydrase IX (CA IX) is a well-established biomarker for aggressive cancers, including TNBC. This study reports, for the first-time, the inhibitory activities of a series of steroidal and non-steroidal sulfamate derivatives against CA IX in comparison to the ubiquitous CA II, with some compounds demonstrating 100-200 fold selectivity for CA IX over CA II. X-ray crystallographic studies of four of the most promising compounds reveal isoform specific residue interactions responsible for the high specificity.

Introduction

Despite advances in cancer diagnosis and treatment, breast cancer remains a serious health issue for women. Nearly 12% of women develop breast cancer during their lifetime, with over 40,000 fatalities each year in the United States (breastcancer.org). While therapies for some selective breast cancers have been successful, patients with triple negative breast cancer (TNBC) have no currently available targeted therapies. This is because TNBC lacks the targeted receptors for estrogen, progesterone and human epidermal growth factor receptor 2 (HER2).^{1,2} TNBC constitutes 15% of breast cancers diagnosed and is associated with a poor prognosis and higher relapse rates compared to luminal and HER2 breast cancers.² Hence, there is a need for new targets for TNBC therapies.

Carbonic anhydrases (CAs) are zinc-containing metalloenzymes that catalyze the reversible hydration of carbon dioxide and water to bicarbonate and a proton.³ There are 15 CA isoforms in humans which are involved in numerous physiological processes such as pH regulation, respiration and blood homeostasis.⁴ Recently, carbonic anhydrase IX (CA IX) has been identified as a potential therapeutic target for TNBC.^{2,5,6} Usually, CA IX expression is limited to the GI tract to maintain an acidic pH. However, under hypoxic tumor conditions CA IX is significantly overexpressed. Tumor hypoxia is caused by the lack of O₂ supply due to the inability of the local vasculature to develop with the growing tumor.⁷ Without oxygen present, the tumor cell shifts from the mitochondrial oxidative phosphorylation pathway to utilizing anaerobic glycolysis, a process termed the Warburg effect.^{7,8} This results in the production and export of lactic acid into the tumor's extracellular environment. To compensate for this decrease in extracellular pH, cancer

cells activate hypoxia-inducible factor 1 (HIF-1 α) that induces its intracellular signaling pathway and causes the upregulation of numerous genes, including CA IX.⁹

CA IX is a transmembrane multi-domain enzyme consisting of a small intracellular domain, a helix transmembrane domain, a highly conserved CA catalytic domain, and an extracellular proteoglycan (PG) domain.¹⁰ Under physiological conditions CA IX exists as a homodimer, although its monomeric truncated catalytic domain is a functional CA. The active site comprises a 15 Å cavity, where a zinc is coordinated by three histidines and a zinc-bound solvent (ZBS).^{4,10} The active site cavity is divided into two distinct sides, a hydrophobic and a hydrophilic side. The hydrophobic side is lined by residues; 91L, 121V, 131F, 135V, 141L, 143V, 198L, 202P, 204A, 207V, 209W (CA II numbering/sequence). These residues are important for the movement of CO₂ in and out of the active site, while, the hydrophilic side is lined by residues; 62N, 64H, 65S, 67Q, 69T, 92Q, 199T, 200T (CA II numbering/sequence). These residues are responsible for stabilizing an ordered water network, involved in the movement of protons in and out of the active site.^{3,10}

The PG and intracellular tail domains make crystallization of the full-length CA IX difficult, due to the large number of exposed hydrophobic residues. Therefore, a soluble CAIX mimic (CAIX_{mimic}) has been developed for structural studies.^{10,11} This CA IX_{mimic}, consists only of the catalytic domain, constructed from a CA II scaffold (a CA isoform that has been shown to be easily crystallized) where the active site residues have been replaced to those of CA IX. The amino substitutions are: Ala65Ser; Asn67Gln; Glu69Thr; Ile91Leu; Phe131Val; Lys170Glu; and Leu204Ala. These residues, while different between the CA II and IX isoforms, retain similar charge or hydrophobicity. One significant difference between the two isoforms is the more solvent “open” active site of CA IX due

to the Phe131Val difference with CA II.^{4,12} The CA IX_{mimic} construct was used for all structural studies.

Steroid sulfatase (STS) is an emerging therapeutic target for the endocrine therapy of hormone-dependent diseases and catalyzes hydrolysis of estrogen sulfate to estrogen.¹³ Development of the aryl O-sulfamate pharmacophore as a key structural motif in drug discovery for STS has led to steroidal and non-steroidal drugs entering many clinical trials, for example STX64/Irosustat, with promising results in oncology and women's health.^{14,15} This "STX" sulfamoylated series of drugs, some of which are employed here, were designed as STS inhibitors and multi-targeting agents and, in many cases, were derived initially from a classic four-ring steroid template; later these were superseded for oncology by very potent and non-estrogenic non-steroidal derivatives such as Irosustat, which has to date been evaluated clinically in hormone-dependent breast cancer, endometrial cancer and prostate cancer. A steroid sulfamate is also currently in clinical trials in endometriosis.^{14,15} Other non-steroidal STS inhibitors are also currently being developed.¹⁶ Another steroidal bis-sulfamate, the non-estrogenic STX140 (3,17 β -Bis-sulfamoyloxy-2-methoxyestra-1,3,5(10)-triene), has also shown great promise as a potential cancer therapy in the hormone-independent setting.^{14,15,17,18} It competes *inter alia* at the colchicine binding site of tubulin, leading to microtubule depolymerization and thus cell cycle arrest and apoptosis^{19–21}. It has also been proven to inhibit HIF-1 α accumulation in the nucleus, causing down-regulation of CA IX mRNA expression.¹⁹ STX140 outperformed the chemotherapy drug Paclitaxel, which also disrupts microtubule growth, reducing tumor size and preventing metastasis in mouse models better than Paclitaxel. In addition, it was shown to have only mild side effects, when compared to

clinically used anti-mitotic agents²² and is active in xenografts derived from patients who failed on docetaxel therapy, showing promise for metastatic triple negative breast cancer.²³ Due to the anti-angiogenic properties of STX140, it was hypothesized that these inhibitors may have a “double hit” therapeutic effect, targeting both microtubule polymerization and inhibiting CA IX.¹⁹

Some later non-steroidal sulfamate derivatives were also developed using pharmacophore translation and good *in vitro* and *in vivo* activity was observed on cancer cell lines.^{24–31} Recently, one member of a class of quinazolinone-based non-steroidal agents with excellent activities was studied crystallographically in atomic detail in complex at the colchicine binding site of tubulin, the first example for such a sulfamate ester.³² Steroid sulfamates and derivatives have been evaluated extensively against CA II and modeled to dock into the CA IX active site.^{33,34} Actual complexes, however, have yet to be elucidated for CA IX, although some crystallographic studies have been performed on CA II with Irosustat and mainly steroidal agents, and on CA II with non-steroidal dual aromatase-sulfatase inhibitors.^{35–37}

Here we report kinetic and docking studies for a series of compounds (Figure 1) against both CA II and CA IX, with multiple compounds exhibiting CA IX specificity. CA II is ubiquitously expressed throughout the body and is therefore used as an off-target comparison for determining CA IX specificity. Four inhibitors were then further studied for structure activity relationships (SARs) through X-ray crystallography and isothermal titration calorimetry (ITC). STX140 and three other derivatives were complexed with CA IX to determine the important sites of interaction that might promote specificity. Of these compounds, the parent STX140 is an estradiol bis-sulfamate with a four membered ring

system and an *ortho*-methoxy group adjacent to the phenyl 3-*O*-sulfamate. Two non-steroidal inhibitors, STX2484 and STX2845, were subsequently developed to mimic STX140 without the rigid steroidal ring system. Instead, these compounds have a two-ring steroidal AB mimic motif connected to the phenolic sulfamate group, with a third ring attached *via* a methylene linker, thus allowing more rotational freedom. The related steroidal STX49 was also synthesized as a simple estradiol bis-sulfamate, mimicking STX140 but without the *ortho*-methoxy group.

Results and Discussion

Kinetic Studies

Stopped flow kinetic analysis of each STX ligand showed efficient inhibition of both CA IX and the off-target CA II at μM concentrations. Of the steroidal subset of STX molecules, STX738 exhibited the highest CA IX K_i value of $1.18 \mu\text{M}$ while the others all showed K_i s below 750 nM with STX641 being the next worse. This is most likely due to the lack of the aryl sulfamate motif. STX140 and STX243 are both bis-sulfamates, with a sulfamate group in the 3- and 17β position. STX641 lacks the cyclopentane ring sulfamate, which is replaced by a mimicking cyanomethyl group, but it still retains a 3-position aryl sulfamate. A bis-sulfamate scaffold seems to be the best candidate for CA IX inhibition among the steroidal class of molecules. It is also likely, perhaps not too surprisingly, that the ligands preferentially bind through the aryl sulfamate, as the K_i s increase in its absence. The non-steroidal subset of molecules also showed efficient inhibition of both CA isoforms with CA IX K_i s ranging from $1.02 \mu\text{M}$ to 2 nM . By comparing the structures of the compounds with the range of K_i s, it becomes apparent that the

addition of more polar groups to the tail portion of the molecules results in higher K_i s. STX2182 and STX3469 both introduce more polar groups to the flexible single ring. Likely, this pendant ring region of the molecule is interacting with the hydrophobic face of CA II and CA IX; thus, adding polar groups disfavors binding. Unsurprisingly, the non-sulfamate subset of ligands exhibited the least efficient inhibition of both CA isoforms. Traditional CA inhibitors utilize a sulfonamide or sulfamate group to bind to the catalytic zinc and block the active site. Without these high affinity binding groups inhibitor K_i s greatly increase. Overall, STX140, STX2484, and STX2845 exhibited the highest inhibitory efficiency, with K_i values of 70, 80, and 2 nM, respectively (Table 1). Kinetic data in full can be seen in Supplemental Material (Figure S1)

While K_i s are important, when designing a CA IX-selective inhibitor, the most crucial kinetic constant is the selectivity ratio. This ratio is an inhibitor's CA II K_i divided by its CA IX K_i . While all inhibitors exhibited efficient K_i s, only a few could be classified as CA IX-specific (Table 1). The steroidal subset of STX inhibitors all reported low selectivity ratios under 10 with STX243 being the most CA IX specific. The non-sulfamate class also showed poor selectivity, with ratios of 7 and 3 for STX2839 and STX2480 respectively. The non-steroidal sulfamates, however, demonstrated impressive CA IX specificity, with STX2845 exhibiting a ratio of 202. By comparing such ratios to structure for each molecule, important motifs for selectivity are elucidated. The three ligands with the highest CA IX selectivity are STX2484, STX2848, and STX2845, with ratios of 104, 155, and 202 respectively (Table 1). These three molecules contain a tri-methoxyphenyl group in the flexible tail portion. The CA II active site is crowded compared to the CA IX active site,

due to the F131 in CA II vs a V131 in CA IX. Likely, this tri-methoxyphenyl motif clashes with the F131 in CA II, resulting in unfavorable steric interactions. In CA IX, however, the V131 provides more room for binding, resulting in stabilizing interactions with the group. Another important motif is the methoxy group located *ortho* to the zinc binding sulfamate. All of the non-steroidal molecules have this *ortho*-methoxy motif, except for STX2845. STX2845 exhibited the best K_i and the best selectivity ratio is the best of the inhibitors. This suggests that an *ortho*-methoxy motif might result in destabilizing interactions within the active site. It is likely that the methoxy group makes the inhibitor too bulky to bind well, causing more solvent displacement and thus decreasing inhibitory efficiency.

Steroidal bis-sulfamate STX49, without the aryl 2-methoxy group of STX140, was subsequently synthesized to probe the effects of the *ortho*-methoxy group. Since STX2845 showed the most promising kinetic data, the *ortho*-methoxy group in the parent compound STX140 was deleted to generate a related ligand that potentially might outperform STX2845.

In Silico Modeling

All STX inhibitors except STX49 were docked into CA II and CA IX. Each inhibitor bound, as expected, with the sulfamate group interacting with the catalytic zinc (Figure 2A). Grid scores were used as a measurement of how well each molecule fits into the active site and as a prediction of the ΔG of binding, more negative numbers indicating better binding. Grid scores for each compound ranged from -90 to 0 for CA II, with STX2480 predicted to be the best binder, and STX140, STX2182, and STX2484 predicted not to bind to CA II at all. Grid scores for CA IX ranged from -100 with STX3209

to -45 with STX2480 (Figure 2). All compounds were shown to bind with moderate affinity to CA IX.

To predict which compounds would be ideal for SAR studies, the docking grid scores and log K_i s were plotted against each other, to generate an ideal inhibitor graph (Figure 2B). The ideal ligand would exhibit a negative $\ln(K_i)$ (indicative of a low K_i) and highly negative grid score (indicative of a high affinity inhibitor). Therefore, this plot should show the best inhibitors under the x-axis and to the left of the y-axis. The *in silico* docking scores predicted the non-sulfamate STX2480 to be the best CA II binder. This of course was proven false by the kinetic data, with the non-sulfamate class of inhibitors reporting the highest K_i s. While a powerful tool, docking software is still limited by solvation energies, metal interactions, and other unaccounted variables of binding. Docking was used in conjugation with K_i s to get a prediction as to which inhibitors would be the most specific to CA IX. As shown in figure 2B, the non-steroidal sulfamate class of inhibitor was shown to be very specific to CA IX and bind with high affinity. The plot clearly shows a trend of non-steroidal molecules being the best binders in CA IX and worst in CA II.

CA IX Inhibitor Complexes

After preliminary kinetics and docking data, four of the inhibitors were further studied using X-ray crystallography. STX140, STX2484, and STX2845 were chosen due to their low K_i s, high selectivity ratios, and grid scores calculated from the in-silico docking (Figures 2 & 3). STX49 was also chosen due to the promising absence of the *ortho*-methoxy group, mimicking that in STX2845. The CA IX inhibitor complexes formed

monoclinic $P2_1$ crystals that diffracted X-rays to maximal resolutions from 1.9 to 1.6 Å (Table 2). Difference electron density maps clearly showed that the ligands had bound in the active site (Figure S2A). As expected, all four inhibitors were tethered *via* their sulfamate group to the zinc, displacing the ZBS (Figure 4). The final refinement statistics for the structures determined were typical for their resolution (Table 2). Of note, previously reported CA IX_{mimic} crystal structures have achieved higher ordered diffraction, better than 1.3 Å resolution. The lower resolutions for these studies are most likely a consequence of the high concentrations of DMSO used during co-crystallization, the DMSO affecting both the protein stability and crystal formation, resulting in higher mosaicity. Despite this, the structures are all within acceptable statistical ranges (Table 2). Occupancies for all inhibitors were determined to be 1.00 indicating binding in every unit cell. For CA-inhibitor complexes, β -factor, or thermal motion within the crystal lattice, was observed to be within acceptable values. β -factors for side chains ranged from 22-28 Å² while the inhibitors ranged from 24-53 Å². β -factors for ligands are similar to side chain values indicating tight inhibitor binding.

Current X-ray crystallography usually takes advantage of cryo-cooling to protect crystals from radiation damage. As data are collected, the X-rays can lead to the formation of free-radicals, which in turn causes decay in the lattice causing poor diffraction and therefore reduced quality. Synchrotrons and in-house X-ray sources take advantage of liquid nitrogen cooling to prevent heating within the lattice thus leading to higher quality diffraction over time. Cryo-cooling was initially attempted with the CA IX-STX complexes, however crystals became brittle and cracked upon freezing resulting in poor data. Utilizing room-temperature diffraction with a low energy in-house X-ray source, co-crystals of CA

IX and each inhibitor showed high resolution diffraction and high quality statistics. While the possibility of radiation damage is greater with room-temperature data collection, it is still a viable technique when cryo-cooling is not an option. Low β -factor values for main and side chain atoms indicate high quality data throughout collection and thus minimal radiation damage.

STX49

For STX49 (PDB: 6e8P, Figure 4A) the observed omit map electron density was weaker than for the non-steroidal inhibitors STX2845 and STX2484, possibly indicating lower binding occupancy and/or weaker binding (Figure S2A, map contoured at 0.7σ). This observation was reinforced further by the fact that only 66% of the inhibitor made contact with active site residues, meaning that 34% was solvent exposed (Table 3). The sulfamate group was stabilized via two hydrogen bonds with Thr199 and Thr200 (distances of $2.8\text{\AA}$), while the fused rings exhibited van der Waals interactions with Val121, Val131, Leu198, and Pro202 (Figure 5A). Based on the crystal structure interactions the estimated ΔG for the complex formation was determined to be -16.3Kcal/mol (Table 3).

The ITC measurements confirmed the weak binding of STX49, as a K_d value could not be directly calculated, however extrapolation of the ITC plots provide an estimated K_d of ~5 mM in CA IX, while no binding constant could be determined for CA II (Table 3).

STX140

STX140 (PDB: 6E8X, Figure 4B) is the parent compound of the series, and inhibits microtubule polymerization. STX140 is chemically similar to STX49, with just the addition of an *ortho*-methoxy group on the phenyl sulfamate. This group is critical in the parent endogenous steroid 2-methoxyestradiol to abolish estrogenicity. The observed omit map electron density for STX140 was the least defined of the four inhibitors studied (Figure S2B, map contoured at 0.7σ). This is most likely due to the methoxy group causing steric hindrance within the active site, causing energetically unfavorable conformational geometry on STX140 and an unfavorable positioning (Figure 4B). However, with the addition of the methoxy group to STX49, an additional hydrogen bond was viable.

STX140 had the same Thr199 and Thr200 hydrogen bonds as STX49 (distances of $2.7\text{\AA}$) through the sulfamate group, an additional hydrogen bond created by the added methoxy moiety (distance of $2.8\text{\AA}$), and it also exhibited extensive van der Waals interactions with Gln92, Val121, Val131, Val135, Leu141, Val143, and Leu198 (Figure 4B). The addition of the extra hydrogen bond would be estimated to stabilize the complex by $\sim -1\text{Kcal/mol}$. This matches the measured ΔG upon complex formation of STX140 to CA IX to be -17.2 Kcal/mol , which is -0.9 Kcal/mol better than STX49 (Table 2). This observation validates that the additional hydrogen bond, and therefore the *ortho*-methoxy group improves binding affinity for the steroidal inhibitors.

STX2484

STX2484 (PDB: 6E91, Figure 4C) is a non-steroidal derivative of STX140 with a rotatable carbon linker between the two ring systems. However, it retains an *ortho*-methoxy group, similar to STX140. The methylene linker resulted in more rotational

freedom of the inhibitor, allowing it to sample multiple rotational configurations during binding to minimize its Gibbs free energy (Table 3 & Figure 5A). STX2484 exhibits a 90° twist compared to the steroidal STX49 and STX140 and the non-steroidal derivative STX2845 (Figure 5A & B). As seen with STX140, it forms hydrogen bonds with Thr199 and Thr200 (distances of 2.9 and 2.7 Å, respectively) and a somewhat weaker hydrogen bond with the methoxy moiety (distance of 3.3 Å). STX2484 also exhibited van der Waals interactions with His64, Gln92, Val121, Val131, Gly132, Val135, Leu198, and Pro202, (Figure 4C). The observed omit map electron density for STX2484 was also the most defined of the four inhibitors (Figure S2C, map contoured at 1.0 σ). The flexibility of the non-steroidal scaffold allows the methoxy moiety to rotate and fit into the small cavity near the zinc, where residue Gln92 helps to stabilize it. It is worth noting that Gln92 may form an additional hydrogen bond with this group. This stabilization, paired with an added hydrogen bond from the Thr residues, results in high binding affinity.

The predicted ΔG of STX2484 CA IX complex formation was calculated to be -18.4 Kcal/mol, the best of the STX series subjected to SAR evaluation. Similarly to STX140, this is likely due to the addition of a hydrogen bond *via* the introduction of the methoxy group. STX2484 also had the highest interaction percentage of solvent buried surface of 71%, maximizing van der Waals interactions within the active site (Table 3). While STX2484 showed the best calculated ΔG of complex formation and the highest area of interaction, it did not exhibit the lowest K_i and K_d (Table 1 & Table 3). While an efficient means of predicting binding, the estimated thermodynamics from ITC are still an estimation and should be accompanied by other experiments.

STX2845

STX2845 (PDB: 6E92, Figure 4D) has a similar structural formula to STX2484 being a non-steroidal derivative, but lacking the *ortho*-methoxy group. Also, similarly to STX2484, STX2845 had well-defined omit map electron density (Figure S2D, map contoured at 1.0σ). As seen for the other STX inhibitors, STX2845 hydrogen-bonded with Thr199 and Thr200, similarly to STX49 (distances of 2.7 and 2.8Å, respectively), but it lacks the methoxy group and therefore the additional hydrogen bond as in STX140 and STX2484. STX2845 exhibited only hydrophobic van der Waals interactions with active site residues Leu91, Val121, Val131, Gly132, Val143, and Leu198 (Figure 4D).

STX2845 had the poorest calculated ΔG for binding to CA IX of the four inhibitors, -16.2 Kcal/mol, and this is probably a consequence of the low buried surface area of 66% and the omission of the hydrogen bond of the methoxy moiety. The program used to calculate the ΔG of binding weighs heavily on the number of hydrogen bonds formed but largely ignores the zinc in the active site. Binding to an active CA isoform is difficult to predict and measure due to the force fields induced by the zinc ion. As seen in Tables 1 and 2, STX2845 was shown to be the best inhibitor with the highest specificity, despite the predicted thermodynamic values.

All the drugs in the STX series showed preference for binding CA IX over CA II in both K_i and K_d (Tables 1 & 3). This likely due to bulkier groups in the active site of CA II than CA IX. The CA II isoform has multiple differences compare to CA IX in active site residues; the most notable one being Phe131. This residue most likely hampers inhibitor access to the active site by inducing steric hindrance blocking binding. However, in CA IX this residue is a valine opening up the active site. Of the four inhibitors studied in the

SAR experiments, STX2484 showed the greatest CA isoform specificity with a 7-fold specificity for CA IX over CA II when comparing binding affinities. However, STX2845 has a 2-fold increase in binding affinity for CA IX, while also having the greatest CA IX specificity in K_i .

The STX series of inhibitors explored the effect of inhibitor conformational flexibility and design aspects of CA inhibitors; a fused steroid ring vs linked ring structures and the effect of the addition of a methoxy group placement near the zinc.

Conformational flexibility makes the non-steroidal inhibitors better binders to CA IX. The steroidal class have K_d s at least 20-fold higher than the more flexible non-steroidal inhibitor STX2484 (Table 3), likely due to the flexibility that the methylene linker adds to STX2484 and STX2845. STX49 and STX140 possess a fused ring structure, making them rigid and bulky. These molecules are very limited in flexibility and thus can only sample a small amount of binding modes compared to the non-steroidals. The result is a higher binding energy, leading to the steroid derivatives not binding as tightly. The more open system of the non-steroidals allows them to rotate and sample more conformations upon binding. More energetically favorable conformations are thus achieved, explaining the better binding affinities (Figure 5B, Table 3).

The addition of the methoxy group seems to have different effects dependent on a given inhibitor. For the steroidal class, STX49 and STX140, it seems that the methoxy substituent allows tighter binding, providing an additional hydrogen bond and also increasing solubility. However, the opposite is observed for STX2484 and STX2845. While the methoxy group does lead to an additional hydrogen bond in STX2484, its binding affinity is 7 fold lower than STX2845. Likely, the methoxy group aids the solubility

of the steroidal inhibitors allowing more compound to be in solution to bind to the active site. In turn, this may also mimic the effects of better binding as the availability of the inhibitor in solution increases. However, in the case of the non-steroidal compounds, that are already much more soluble, it seems that binding with the methoxy group results in a less energetically favorable conformation, despite the additional hydrogen bond, by forcing the compound into an unfavorable pocket. The compound must twist in conformation when the *ortho*-methoxy group is present, resulting in a decrease in affinity for STX2484 in CA IX.

Due to differences in steric hindrance, each of the ligands occupies a different, slightly rotated position in the active site to maximize energy-stabilizing interactions (Figure 5A). The inhibitors bind similarly based on the presence of the methoxy group. STX49 and STX2845 bind planar to each other, with their first two rings superimposable. However, with the methoxy group present, the inhibitor has to “find” a conformation to bind to the zinc. Therefore, it samples multiple binding modes before arriving at a local energy minimum. STX140 rotates approximately 40° from STX49 and STX2845 (Figure 5B). This places the methoxy group in an area of charged residues that stabilize the alternate configuration. STX2484 binds similarly, twisting ~90° away from the other three inhibitors to place its methoxy group in a polar cavity (Figure 5A & B).

STX49 showed the lowest solubility of the inhibitors tested in the SAR studies and, as such, a high DMSO concentration was required for experiments. Most proteins are unstable in DMSO and CA has been reported to decompose at concentrations above 10%. Therefore, the limit of ITC was reached due to STX49 poor solubility and the K_d could not be calculated.

Conclusions

A series of 11 sulfamate and 2 non-sulfamate phenolic ligands were evaluated for their binding and inhibition of CA II and CA IX. Named the “STX series”, these compounds are structural relatives to the compound STX140, which could potentially be a cancer therapy in a hormone-independent setting.^{14,15,17,18} These molecules were classified under three subsets: steroidal, non-steroidal and non-sulfamates (Figure 1). All ligands studied were shown to inhibit CA II and CA IX through the use of a stopped flow kinetic apparatus monitoring the amount of ¹⁸O exchange between CO₂ and water. The non-steroidal subset in particular showed the best inhibition, with STX2845 possessing a K_i of 2 nM (Table 1).

Molecular docking was then performed using the UCSF Dock 6 program. Each ligand was docked into the active site of CA II and CA IX and assigned a grid score based on how well it interacts with active site residues. Scores ranged from 0, indicative of no binding, to -100, meaning tight binding. By plotting K_i vs the grid score for every compound, the best candidate inhibitors were chosen for further SAR studies. STX140, STX2484, and STX2845 were explored in additional studies due to their low K_is, high selectivity ratios, and predicted grid scores. STX49 was designed to examine the effects of the *ortho*-methoxy motif (Figure 2 & 3).

X-ray crystallography was performed on these four compounds in complex with CAIX_{mimic}, followed by ITC. X-ray crystallography confirmed that all of these compounds bound through the aryl 3-O-sulfamate moiety, confirming observations in the kinetic studies. Val121, Val131, Thr199, and Thr200 were identified as important residues for inhibitor binding and Val 121 and 131 may be crucial for CA IX specificity (Figures 4-5).

Of the STX inhibitors in the SAR studies, STX2845 and STX2484 were observed as the most effective and STX49 the poorest. Comparing K_i s between CA II and CA IX, STX2484 showed a high CA IX specificity of ~100-fold preference for CA IX. STX2845 showed a similar preference of ~200 indicating its high specificity. Furthermore, ITC experiments confirmed tighter binding with CA IX for both of these inhibitors. STX2484 and STX2845 exhibit cLogP values of 1.43 and 1.51 respectively. These values indicate high solubility and infer high bioavailability (Figure 3). These two non-steroidal inhibitors are the lead compounds emerging from these experiments and should be further derivatized to lead to more specific compounds. Due to limited solubility, STX49 binding could only be measured via X-ray crystallography with high concentrations of DMSO. ITC was unable to detect complex formation between STX49 and CA IX and the stopped flow kinetic assay could not detect inhibition. The stopped-flow kinetic assay and ITC experiments are limited by organic solvent concentrations and therefore low solubility compounds cannot be measured accurately with these methods. STX49 exhibits a cLogP value of 2.32, a significant decrease in solubility compared to the non-steroidal compounds. This insolubility would translate to poor bioavailability *in vivo* thus making STX49 a poor therapeutic. The other steroidal inhibitor STX140 in contrast performed much better in SAR experiments, showing μ M binding affinities, a nM K_i , and a lower ΔG in binding energy. The added methoxy group decreased STX140 cLogP value to 2.23, allowing higher solubility and therefore a higher bioavailability, something already established *in vivo*.^{17,18}

CA II is the main off-target destination for CA IX targeted drug therapy, as it is expressed ubiquitously in a large concentration in the cytoplasm of most cells. CA IX,

however, is expressed at relatively low concentrations on the extracellular surface of cancer cells. Traditionally, steroid molecules can efficiently transverse the cellular membrane due to their hydrophobic nature. Steroids have a cellular membrane permeability coefficient of 10^{-3} to 10^{-4} cm/s, similar to that of water, 3.4×10^{-3} cm/s.^{38,39} However, molecules with cLogP values higher than 5 are known to be cell membrane impermeable.⁴⁰ Hence, designing slightly insoluble inhibitors that have difficulty crossing the cellular membrane could prove beneficial for the STX series, as well as for any CA IX targeted therapy in the future. Therefore, slightly insoluble non-steroidal derivatives may prove to be even more selective for CA IX *in vivo*.

STX140 and the other twelve inhibitors are intriguing for potential oncology applications as some of them have been shown to exhibit a ‘double hit effect’, stopping both microtubule polymerization and being both *pro*-apoptotic and anti-angiogenic. Multiple targeting agents are currently growing in popularity with double and even triple targeting compounds in pharmaceutical pipelines.⁴¹ Inhibiting CA IX activity in a targeted and selective fashion over CAII could thus add an additional advantage, linked through addressing pH dysregulation, an essential mechanism for cancer cell survival. Further derivatives should be synthesized to explore the effects of adding diverse chemical motifs to these compounds. The *ortho*-methoxy group in particular should be closely examined, as its effect on binding was different between the steroidal vs non-steroidal compounds. Invasion and migration assays with CA IX-expressing cell types would also be a great way to determine the anti-angiogenic effects of these inhibitors. Overall, these drugs represent promising candidates targeted to TNBC.

Experimental

1. Compounds

STX49 (3,17 β -Bis-sulfamoyloxyestra-1,3,5(10)-triene), STX140 (3,17 β -Bis-sulfamoyloxy-2-methoxyestra-1,3,5(10)-triene), STX243 (3,17 β -Bis-sulfamoyloxy-2-ethylestra-1,3,5(10)-triene), STX641 (17 β -Cyanomethyl-2-methoxy-3-sulfamoyloxyestra-1,3,5(10)-triene), STX738 (3-Hydroxy-2-methoxy-17 β -sulfamoyloxyestra-1,3,5(10)-triene), STX2182 (2-(3,4-Dimethoxybenzoyl)-7-methoxy-6-sulfamoyloxy-3,4-dihydroisoquinolin-1(2H)-one), STX2480 (6-Hydroxy-7-methoxy-2-(3,4,5-trimethoxybenzyl)-1,2,3,4-tetrahydroisoquinoline), STX2484 (7-Methoxy-6-sulfamoyloxy-2-(3,4,5-trimethoxybenzyl)-1,2,3,4-tetrahydroisoquinoline), STX2839 (6-Hydroxy-2-(3,4,5-trimethoxybenzyl)-1,2,3,4-tetrahydroisoquinoline), STX2845 (6-Sulfamoyloxy-2-(3,4,5-trimethoxybenzyl)-1,2,3,4-tetrahydroisoquinoline), STX2848 (($\pm$)-3-Ethyl-7-methoxy-6-sulfamoyloxy-2-(3,4,5-trimethoxybenzyl)-1,2,3,4-tetrahydroisoquinoline), STX3209 (($\pm$)-7-Methoxy-3-methyl-6-sulfamoyloxy-2-(2-chloro-5-methoxybenzyl)-1,2,3,4-tetrahydroisoquinoline) and STX3469 (3-(2,3-Dimethoxybenzyl)-7-sulfamoyloxy-6-methoxy-3,4-dihydroquinazolin-2(1H)-one) were synthesized as described.^{17,24–29,32,42} The compounds exhibited spectroscopic and analytical data in accordance with their structures (Figure 1) and were pure ($\geq 98\%$), as determined by high-performance liquid chromatography.

2. Protein Expression and Purification

CA IX_{mimic} was expressed and purified according to the previously published protocols.^{10,43} Briefly, the CA IX gene-containing plasmid was transformed into

competent BL21 *E.Coli* cells via a standard BL21 transformation protocol that were then transferred to an overnight growth in nutrient rich Luria Broth. Cells were then allowed to grow to an optical density of 0.6 at 600nm in a large scale growth in the presence of selecting antibiotic. The cells were then induced by the addition of 0.5mM isopropyl β -D-1-thiogalactoside (IPTG) and 1mM zinc sulfate and incubated for an additional 3 hours. The addition of zinc during induction aids expression and protein folding. Cells were then lysed using a microfluidizer and CAIX_{mimic} was purified using affinity chromatography with a gravity column and benzylsulfonamide resin. Purity was determined with SDS-Page and protein concentration was determined by UV/Vis spectroscopy at 280nm.

3. Kinetic Studies

CA II and CA IX kinetics and inhibition were studied using a previously published protocol utilizing a stopped flow apparatus and membrane-inlet mass spectrometer to measure the exchange of ^{18}O between CO_2 and water.⁴⁴

4. Docking

The STX series of compounds were docked both to CA II and CA IX using the DOCK 6 program found in the program suite of UCSF.^{34,45,46} Docking was scored and compared between each isoform for twelve of the compounds.

5. X-ray Crystallography

Initial attempts at capturing the STX inhibitors in complex with CA IX_{mimic} using cryogenic conditions were unsuccessful. It is possible that the rapid cooling procedure or

the cryo-protectant hindered the inhibitors binding within the active site. CA IX_{mimic} was successfully co-crystallized with each inhibitor using the hanging drop vapor diffusion method. Crystal trays were set up with 500uL of mother liquor, containing 1.6M sodium citrate and 50mM Tris at a pH of 7.8. Each hanging drop consisted of a 1:1 ratio of 10mg/mL protein to mother liquor. The inhibitors were dissolved in dimethyl sulfoxide (DMSO) to ~1mM and added to the drops to a final concentration of ~100uM.

CA IX_{mimic} co-crystals formed after 3 weeks. A crystal of each inhibitor-complex was wet mounted into quartz capillary tubes, with excess solvent removed, and the tubes sealed with wax. Diffraction data were collected using an in-house X-ray generator at room temperature using an RU-H3R rotating copper anode with a wavelength 1.5418Å. and the X-ray generator running at 50kV and 22mA. Diffraction images were collected on a Rigaku 0.3mm R-Axis VI⁺⁺ image plate detector. Data were collected with a detector distance of 120mm, with 1.0° oscillations, and an exposure time set of 300s and indexed, integrated, merged, and scaled to the P2₁ space group using the program HKL2000 ⁴⁷. Molecular replacement phasing was performed using the software package PHENIX with the PDB entry 3KS3 ⁴⁸. Phases, model refinements, and ligand restraints were also calculated using PHENIX. Further refinement and model building was performed using the interactive modeling program Coot ⁴⁹. Figures were generated in the molecular graphical software PyMol and protein-ligand interactions and bond lengths were determined using LigPlot Plus ^{50,51}. Upon complex determination, PDB Pisa was used to determine surface interactions and estimated free binding energies ⁵².

6. Isothermal Titration Calorimetry

ITC was performed with CA II and CA IX isoforms and each inhibitor to measure relative binding affinities. While preparing protein and inhibitor solutions, it is critical that each solution is identical to prevent heat of solvent dilution (except for drug and protein). To ensure this, the same concentrations of storage buffer and DMSO were used in the drug and protein solutions. A 500 μ M solution of each inhibitor was made in 10% DMSO diluted in CA stabilization buffer (50mM Tris, 100mM NaCl, pH 7.8). CA IX was prepared to 50 μ M diluted in the CA stabilization buffer with 10% DMSO. Experiments were performed on the MICROCAL ITC 200 manufactured by Malvern. 300 μ L of degassed CA IX was loaded into the sample cell of the micro ITC and 60 μ L of the degassed inhibitor solution was loaded into the syringe. The ITC program was set to one 0.2 μ L injection with eighteen subsequent 2 μ L injections of drug, stirring at 700rpm. All experiments were performed at 25°C with a reference baseline heat of 5 μ cal. Due to low heat of binding, inhibitor concentration was increased if data did not indicate saturation. Heat peaks were generated, measured, integrated and used to calculate binding affinities in the microITC analysis software package provided.

Supplemental Information

The Supplemental Information includes: Molecular formula strings for each inhibitor; raw kinetic data for the 12 compounds reported in Table 1; the 2FO-FC electron density maps for each inhibitor; PDB codes STX49:6E8P, STX140: 6E8X, STX2484: 6E91, STX2845: 6E92. Authors will release the atomic coordinates and experimental data upon article publication. Molecular strings formula files are also included.

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Author Contributions

Andring was responsible for X-ray Crystallography and ITC, Dohle and Potter were responsible for inhibitor synthesis, Tu was responsible for kinetic studies, Potter and McKenna were responsible for experimental design and planning. All authors contributed to the writing and editing of the manuscript.

Notes

Authors declare no conflict of interest.

Abbreviations Used

TNBC: triple negative breast cancer, CA: carbonic anhydrase, HER2: human epidermal growth factor receptor 2, HIF-1 α : hypoxia-inducible factor 1, PG: proteoglycan, ZBS: zinc-bound solvent, STS: Steroid sulfatase, SARs: structure activity relationships, ITC: isothermal titration calorimetry, PDB: protein data bank, IPTG: isopropyl β -D-1-thiogalactoside, DMSO: dimethyl sulfoxide

Acknowledgments

We acknowledge the University of Florida Center for Structural Biology for maintaining the X-ray crystallography and biophysical instrumentation facilities. Many of the compounds studied here were originally synthesized with the aid of research funding from Sterix Ltd.

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Tables and Figures

Table 1: CA inhibition study. Measured K_i of each inhibitor complexed with CA II and CA IX..

Inhibitor	CA IX K_i (μM)	CA II K_i (μM)	Selectivity ratio*
STX-140	0.07	0.27	4
STX-243	0.25	2.42	10
STX-641	0.75	1.47	2
STX-738	1.18	10.33	9
STX-2182	0.86	4.44	5
STX-2484	0.08	8.64	104
STX-2845	2.0×10^{-3}	0.34	202
STX-2848	0.45	69.64	155
STX-3209	0.26	5.31	20
STX-3469	1.02	4.73	5
STX-2839	90.59	678.96	7
STX-2480	18.54	51.94	3

* K_i of CA II / K_i of CA IX

Table 2: Crystallographic data statistics

Inhibitor	STX49 (PDB:6E8P)	STX140 (PDB:6E8X)	STX2484 (PDB:6E91)	STX2845 (PDB:6E92)
Space Group	P2 ₁	P2 ₁	P2 ₁	P2 ₁
Cell Dimensions: <i>a</i> , <i>b</i> , <i>c</i> , β (Å, °)	42.6, 41.7, 72.6, 104.0	42.5, 41.7, 72.7, 104.0	42.5, 41.7, 72.7, 103.9	42.5, 41.7, 72.6, 104.0
Resolution (Å)	18.6 - 1.9 (1.97 - 1.9)	18.9 - 1.6 (1.65 - 1.6)	18.9 - 1.8 (1.86 - 1.8)	29.3 - 1.8 (1.83 - 1.8)
Total Reflections	27,295	32,736	31,904	31,356
I/ σ	12.7 (2.1)	21.2 (2.5)	20.8 (3.1)	24.1 (3.2)
Redundancy	4.4 (4.4)	3.8 (3.8)	6.2 (6.2)	3.6 (3.6)
Completeness (%)	99.8 (98.2)	100 (100)	97.1 (97.7)	93.2 (95.5)
R _{sym} ^a (%)	10.8 (66.1)	5.9 (38.7)	8.7 (57.9)	4.8 (36.4)
R _{cryst} ^b (%)	14.8 (25.0)	16.6 (25.0)	14.0 (19.2)	14.5 (20.4)
R _{free} ^c (%)	19.1 (26.7)	18.9 (28.4)	17.4 (28.5)	18.1 (23.6)
R _{pim} ^e (%)	5.8 (35.8)	3.4 (27.6)	3.7 (25.0)	3.0 (22.6)
Residue Nos. ^f	4 - 261	4 - 261	4 - 261	4 - 261
# of Protein Atoms	2087	2145	2116	2094
# of Water Molecules	87	95	115	92
% of Ligand Atoms	28	30	30	28
Ramachandran Stats (%) Favored, Allowed	96.9, 3.1	97.4, 2.7	97.3, 2.7	97.7, 2.3
Avg. B Factors (Å ²): Main-, Side-chain, Solvent, Inhibitor	21.2, 27.6, 36.2, 31.5	14.2, 21.6, 26.4, 52.7	20.0, 27.5, 33.7, 24.1	19.3, 26.4, 32.6, 50.3
RMSD for Bond Lengths, Angles (Å, °)	0.007, 1.169	0.010, 1.740	0.009, 1.426	0.008, 1.557

$$^a R_{\text{sym}} = (\sum |I - \langle I \rangle| / \sum \langle I \rangle) \times 100.$$

$$^b R_{\text{cryst}} = (\sum |F_o - F_c| / \sum |F_o|) \times 100.$$

^cR_{free} is calculated in the same way as R_{cryst} except it is for data omitted from refinement (5% of reflections for all data sets).

$$^d R_{\text{pim}} = [(\sum \sqrt{1/N - 1}) \sum |I - \langle I \rangle| / \sum \langle I \rangle] \times 100.$$

^eChains numbered relative to hCA IX mimic.

Values in parentheses correspond to those of the highest-resolution shell.

Table 3: STX binding statistics

STX Inhibitor	Surface Interface Interaction Area (Å ²)	Interaction Percentage	Estimated ΔG (Kcal/mol)	Displaced Waters	Kd CA IX (nM)	Kd CA II (nM)
<p>STX49</p>	390	66%	-16.3	10	N/A*	N/A
<p>STX140</p>	400	64%	-17.2	9	605	3750
<p>STX2484</p>	470	71%	-18.4	13	240	1620
<p>STX2845</p>	410	66%	-16.2	11	35	70

*N/A: Inhibitor insoluble

Figure Legends

Figure 1: Structural formula of STX inhibitors: Ligands are grouped according into subsets: Steroidal, Non-Steroidal, and Non-Sulfamate.

Figure 2: *In silico* Docking of STX inhibitors to CA II and CA IX: A) Docking results of inhibitors complexed with CA IX PDB:5DVX (CA II not shown). B) Grid score correlated to experimentally measured K_i . X-axis: $\ln(K_i)$, Y-axis: Grid score.

Figure 3: Inhibitors used for X-ray crystallography: These inhibitors were selected for further SARs due to low K_i s and favorable *in silico* docking scores.

Figure 4: Crystallographic inhibitor interactions with CA IX_{mimic}: A) STX49 (green) PDB:6E8P, B) STX140 (cyan) PDB:6E8X, C) STX2484 (yellow) PDB:6E91, D) STX2845 (pink) PDB: 6E92. Residues are as labeled. G132 (not shown) interacted with STX2484 and STX2845. Dashed lines represent hydrogen bonding all distances given in Å.

Figure 5: Surface rendition of CA IX_{mimic} complexed with STX Inhibitors superimposed: A) Insert, complete surface of CA IX. STX49 (green) PDB:6E8P, STX140 (cyan) PDB:6E8X, STX2484 (pink) PDB:6E91, and STX2845 (yellow) PDB: 6E92. Hydrophobic region of active site in orange and hydrophilic region in violet. B) Rotation of inhibitors within active site show different binding modes between the inhibitors.

Figure 1

Figure 2

Figure 3

<p>STX49</p> <p>ClogP: 2.32</p>	<p>STX140</p> <p>ClogP: 2.23</p>
<p>STX2484</p> <p>ClogP: 1.43</p>	<p>STX2845</p> <p>ClogP: 1.51</p>

Figure 4

Figure 5